IMMUNIZATION OF NON-HUMAN MAMMALS AGAINST *STREPTOCOCCUS EQUI*

Background of the Invention

1. Field of the Invention

This invention is generally related to antigenic compositions and use thereof for immunization of non-human mammals, e.g. horses, against *Streptococcus equi.*

Streptococcal infections in horses are mainly caused by the species *Streptococcus equi*, which is classified as a Lancefield Group C *Streptococcus* and comprises two subspecies designated *equi* and *zooepidemicus,* respectively.

Streptococcus equi subsp. *equi,* which is virtually confined to horses, is the causative agent of strangles, a world-wide distributed and highly contagious serious disease of the upper respiratory tract of the Equidae. Strangles is one of the most frequently reported equine diseases world-wide and is characterized by fever, nasal discharge, and abscess formation in the retropharyngeal and mandibular lymph nodes. In some cases the disease shows a metastatic course in the body, so called "bastard strangles". The disease has a world-wide distribution and causes great economic losses. Moreover, since strangles is a highly contagious disease, not only infected animals but also all other members of e.g. an afflicted stud must be isolated for as long as up to three months.

S. equi subsp. *zooepidemicus* is considered as an opportunistic commensal often occurring in the upper respiratory tract of healthy horses. However, after stress or virus infection, it can cause a secondary infection, which results in strangles-like symptoms. Moreover, subsp. *zooepidemicus* infects not only horses but also a wide range of other animals, like pigs, dogs, cats, and cows. Even human cases of infection due to subsp. *zooepidemicus* have been reported. This subspecies has been implicated as the primary pathogen in conditions such as endometritis, cervicitis, abortion, mastitis, pneumonia, abscesses and joint infections.

Although it is possible to treat and cure these streptococcal infections with antibiotics, such as penicillin, tetracycline or gentamicin, an effective prophylactic agent that could prevent outbursts of such infections and obviate or reduce the risk for development of resistant strains associated with antibiotic treatment, would be appreciated.

2. Description of the Related Art

However, although many attempts have been made to develop prophylactic agents such as vaccines against *S. equi*, at the present time no efficient vaccines or immunizing preparations are available, neither for the subspecies *equi* nor for the subspecies *zooepidemicus*.

Existing vaccines against strangles are based on inactivated, e. g. heat-killed, or attenuated strains of *S. equi* subsp. *equi* or acid extracts/mutanolysin enriched in M-protein(s), i.e. immunogenic protein(s) produced by *S. equi*. A vaccine against *S. equi* subsp. *zooepidemicus* based on an M-like protein is disclosed in US-A-5,583,014. In WO 87/00436, an avirulent strain of *S. equi* is disclosed for use as a vaccine against *S. equi* that stimulates an antibody response in the nasopharyngeal mucosa after administration thereof to a horse.

Since the previously developed vaccines or immunizing preparations are hampered by side-effects and, moreover, provide insufficient protection, there is a need for efficient and safe prophylactic agents, such as vaccines, that protect against *S. equi* infections and/or prevent spread thereof without giving rise to undesirable side-effects.

It is well known that attachment to eukaryotic cell surfaces is an essential step in the establishment of infection and colonization by bacterial pathogens. Accordingly, streptococcal surface proteins, that interact with and/or bind to different components of the Extracellular Matrix (ECM) or plasma proteins of the host cell, are potential candidates for use as active component(s) for immunizing purposes.

This is illustrated by the vaccines based on M-like proteins mentioned above or disclosed in the literature, i.a. in WO 98/0561. The binding of fibrinogen and complement factor H to M-proteins is assumed to be important for the ability of streptococci to resist phagocytosis by polymorphonuclear leucocytes.

Another mechanism used by streptococci for attachment to host cells involves binding to the ECM component fibronectin (Fn) (Ref. 21, 22). Binding between Fn-binding bacterial cell-surface proteins and immobilized Fn promotes internalization of streptococci by epithelial cells (Ref. 2, 23, 24). Fibronectin is a dimeric glycoprotein found both in plasma and in a fibrillar form in the extracellular matrix. The main function of Fn is to mediate substrate adhesion of eukaryotic cells, which involves the binding of specific cell-surface receptors to certain domains of the Fn molecule. Furthermore, it also interacts with several other macromolecules, such as DNA, heparin, fibrin, and collagen.

Accordingly, Fn-binding proteins from different streptococcal species have been cloned and sequenced previously. For instance, from *S. equi*, one Fn-binding protein has been

cloned and characterized, which is a Fn-binding cell-surface protein of subsp. *zooepidemicus*, that has been designated FNZ (Lindmark et al., 1996, Ref. 9). Another Fn-binding protein from *S. equi* subsp. *equi,* has been cloned and characterized by Lindmark and Guss (1999) (Ref. 12). This latter protein that is designated SFS and its potential use as an active component in a vaccine for protection of horses against strangles are disclosed in WO 00/37496.

In Jonsson et al. (1995) (Ref. 8), a protein designated ZAG has been cloned and characterized from *S. equi* subsp. *zooepidemicus* that mediates binding to the plasma proteinase inhibitor α_2M. It is speculated therein that this protein is similar in function to streptococcal M proteins. This protein, ZAG, is also disclosed in WO 95/07296, where its α_2M-binding properties are indicated. However, immunogenic properties or potential use thereof as an active component in a vaccine for protection of e.g. horses against strangles are not disclosed therein. The gene *zag* encoding ZAG is also disclosed in these references.

A gene that is similar to the aforesaid *zag* gene from *S. equi* subsp. *zooepidemicus* but is present in subsp. *equi* has been described by Lindmark et al. (1999) (Ref. 11) and Lindmark (1999) (Ref .13). This gene is hereafter designated *eag* and encodes a protein designated EAG.

Brief Summary of the Invention

The present invention is based on an antigenic composition comprising at least one antigen that comprises at least one antigenic epitope or antigenic determinant derived from a protein present in one or both of *S. equi* subsp. *equi* and subsp. *zooepidemicus* and use thereof for immunization of non-human mammals against *S. equi* subsp. *equi* and/or subsp. *zooepidemicus*.

The present invention is also directed to a vaccine composition comprising the aforesaid antigenic composition as immunizing component, methods to prepare said antigenic or vaccine composition, methods to induce an immune response against *S. equi* in non-human mammals and methods for prophylactic or therapeutic treatment of *S. equi* infection in non-human mammals. When used generally, the expression "*S. equi*" refers to one or both of subsp. *equi* and subsp. *zooepidemicus*.

According to a suitable embodiment, the present invention is directed to a vaccine that protects equines, such as horses, against strangles.

In the context of infections caused by *S. equi* subsp. *equi*, the expression "non-human mammals" primarily refers to animals belonging to the family Equidae that consists of horses, donkeys and zebras and to hybrids thereof, such as mules and hinnies.

In connection with infections caused by *S. equi* subsp. *zooepidemicus*, the expression "non-human mammals" in addition refers also to other mammals such as cows, pigs, dogs and cats.

Brief Description of the Drawings

In the following, the present invention is described in closer detail with reference to the drawings, where

Fig. 1 shows IgG antibodies developed against FNZN in eight individual mice after intranasal inoculation with *Streptococcus equi* subsp. *equi*.

Fig. 2 shows IgG antibodies developed against SFSC1 in eight individual mice after intranasal inoculation with *Streptococcus equi* subsp. *equi*.

Fig. 3 shows IgG antibodies developed against EAG4B in eight individual mice after intranasal inoculation with *Streptococcus equi* subsp. *equi*.

Fig. 4 shows IgG antibodies developed in mice against FNZN after subcutaneous immunization with FNZN, SFSC1, and EAG4B. Results obtained for seven immunized mice and one non-immunized (▼) mouse are shown.

Fig. 5 shows IgG antibodies developed in mice against SFSC1 after subcutaneous immunization with FNZN, SFSC1, and EAG4B. Results obtained for seven immunized animals and one non-immunized animal (▼) are shown.

Fig. 6 shows IgG antibodies developed in mice against EAG4B after subcutaneous immunization with FNZN, SFSC1, and EAG4B. Results obtained for seven immunized animals and one non-immunized animal (▼) are shown.

Fig. 7 shows IgG antibodies developed in mice after subcutaneous immunization with SEC 2.16. Results from five immunized and five non-immunized mice are shown. (Pre-immune sera gave no response, values closest to the base line with symbols overlapping each other).

The results shown in **Fig. 1-7**, have been obtained with an ELISA test.

Fig. 8 shows the accumulated number of mice that survived or lost less than 15% of weight after subcutaneous immunization with FNZN, SFSC1, and EAG4B followed by challenge with *Streptococcus equi* subsp. *equi* (n= 24).

Fig. 9 shows nasal growth after subcutaneous immunization of mice with FNZN, SFSC1, and EAG4B followed by challenge with *Streptococcus equi* subsp. *equi*. Growth is determined on a scale of 0-3 (n= 24). Mean and SE (Standard Error) are shown. The control group was not immunized.

Fig. 10 shows weight loss of mice after intranasal immunization of mice with FNZN, SFSC1, and EAG4B followed by challenge with *Streptococcus equi* subsp. *equi* (n= 24). The control group was not immunized.

Fig. 11 shows nasal growth after intranasal immunization of mice with FNZN, SFSC1, and EAG4B followed by challenge with *Streptococcus equi* subsp. *equi* (n= 24). Mean and SE are shown.

Fig. 12 shows IgA antibodies against SFSC1 in bronchoalveolar lavage (BAL) and nasal washes (NW) from mice nasally immunized with FNZN, SFSC1, and EAG4B. Results from six animals are shown.

Fig. 13 shows weight loss of mice after intranasal immunization of mice with EAG4B followed by challenge with *Streptococcus equi* subsp. *equi* (n=10). The control group was not immunized.

Fig. 14 shows IgG antibodies in immunized horses developed against EAG4B and FNZN.

Fig. 15 shows the results from a comparison of serum antibody titers from horses with or without a history of strangles.

Fig. 16 shows the presence of IgA antibodies in nasal washings from immunized horses.

Fig. 17 shows antibody titers against EAG4B in horses immunized with EAG4B, FNZN, and SFSC1 as a function of time

Fig. 18 shows the results obtained for mice (n=15) that were immunized with SEC2.16 using Matrix as adjuvant. The number of animals that lost more than 15% of weight or that died is shown as a function of time.

Fig. 19 shows the results obtained for mice that were immunized with SEC2.16 alone (group B (n=5)) or SEC2.16 together with Matrix (group A (n=5)). Serum samples were taken before (denoted pre) and after (denoted imm) immunization.

Detailed Description of the Invention

The present invention is directed to an antigenic composition comprising at least one antigen, wherein said at least one antigen comprises at least part of a protein of *Streptococcus equi* subsp. *equi*, and said at least part of said protein comprises at least one antigenic epitope or antigenic determinant of *Streptococcus equi*, and wherein said protein is comprised of a

protein that is designated EAG and comprises an N-terminal amino acid sequence, which is shown below as SEQ. ID. NO: 1 and is designated EAG4B.

SEQ. ID. NO: 1:

```
1   MALDATTVLE PTTAFIREAV REINQLSDDY ADNQELQAVL ANAGVEALAA DTVDQAKAAL
61  DKAKAAVAGV QLDEARREAY RTINALSDQH KSDQKVQLAL VAAAAKVADA ASVDQVNAAI
121 NDAHTAIADI TGAALLEAKE AAINELKQYG ISDYYVTLIN KAKTVEGVNA LKAKILSALP
```

According to a suitable embodiment of the present invention, said antigenic composition comprises at least one further antigen that comprises at least part of a fibronectin-binding protein of *Streptococcus equi*, said at least part of said protein comprising at least one antigenic epitope or antigenic determinant of *Streptococcus equi*, and wherein said protein is selected from the group consisting of FNZ (EMBL sequence data bank accession number X99995) comprising an amino acid sequence as shown in SEQ. ID. NO: 2 below:

MKTKSFRKVLTTSATCIVLATSFAGGTLRVWAEQLYYGWNDGTRQSSPYFLYVSPKNAPKRE
LKDEYVVYCFNKKLYWPDQWESIYSNFNDIRSPYNDLPVYEKKLGYDGIFKQYAPDYKKDIS
DIASALVAVLSNGYPTNKSQLSTSYHLNNDSSRKVTQLAIWYFSDSLTKEYLKDTGGYNLND
MEKKALDFLISKGEDSKLKSEQSNYSLDIYVYQSGGHDHMKDYQNLLGSTLIPKEPLKPQLG
GFSGHNGNGLSGLEGGSSGSQETNEDGKKGLIGFHGGLSGSEGKRDPLPGLKGEAGAPDTPQ
KPNDPLQGLEGGNSPIVEQNYGSTEGYHGQSGILEETEDTNPPGIILGGSGNVETHEDTRNP
HLMGIGGGLAGESGETTPKPGQTGGQGPVIETTEDTQKGMSGQSGGTIESENTKKPEVMIGG
QGQTIETTEDTQKGMSGQSGGTIESEDTKKPEVMIGGQGQIIDFSENTQSGMSGQSGDTTVI
EDTKKSEIIIGGQGQIIDFSEDTQPGMSGQSGGTTIVEDTKKPTPKPKPAPAPIVNDEKPNK
GTHLPQTSDMKQLTLSIIGAMSMLLVLCLSLFKRPSKKD

and SFS comprising an amino acid sequence as shown in SEQ. ID. NO: 3 below:

MRKTEGRFRTWKSKKQWLFAGAVVTSLLLGAALVFGGLLGSLGG

SSHQARPKEQPVSSIGDDDKSHKSSSDSMVSRPPKKDNLQPKPSDQPTNHQHQATSPS

QPTAKSSGHHGNQPQSLSVNSQGNSSGQASEPQAIPNQGPSQPLGLRGGNSSGSGHHH

QPQGKPQHLDLGKDNSSPQPQPKPQGNSPKLPEKGLNGENQKEPEQGERGEAGPPLSG

LSGNNQGRPSLPGLNGENQKEPEQGERGEAGPPSTPNLEGNNRKNPLKGLDGENKPKE

DLDGKGLSGENDESPKLKDEHPYNHGRRDGYRVGYEDGYGGKKHKGDYPKRFDESSPK
EYNDYSQGYNDNYGNGYLDGLADRGGKRGYGYSYNPD.

Suitably, the afore-mentioned antigenic compositions also comprise at least one further antigen that comprises at least part of an extracellular matrix-binding protein of *Streptococcus equi* and said at least part of said protein comprises an antigenic epitope or an antigenic determinant of *Streptococcus equi* and wherein said protein is comprised of a

protein that is designated SEC and comprises an amino acid sequence as shown in SEQ. ID. NO: 4 below.

SEQ. ID. NO: 4:

1 27

LKQLTKIVSVVLLLVFTLSASLHKVRATNLSDNITSLTVASSSLRDGERTTVKVAFDD
KKQKIKAGDTIEVTWPTSGNVYIQGFNKTIPLNIRGVDVGTLEVTLDKAVFTFNQNIE
TMHDVSGWGEFDITVRNVTQTTAETSGTTTVKVGNRTATITVTKPEAGTGTSSFYYK
TGDMQPNDTERVRWFLLINNNKEWVANTVTVEDDIQGGQTLDMSSFDITVSGYRNE
RFVGENALTEFHTTFPNSVITATDNHISVRLDQYDASQNTVNIAYKTKITDFDQKEFA
NNSKIWYQILYKDQVSGQESNHQVANINANGGVDGSRYTSFTVKKIWNDKENQDGK
RPKTITVQLYANDQKVNDKTIELSDTNSWQASFGKLDKYDSQNQKITYSVKEVMVP
VGYQSQVEGDSGVGFTITNTYTPEVISITGQKTWDDRENQDGKRPKEITVRLLANDA
ATDKVATASEQTGWKYTFTNLPKYKDGKQITYTIQEDPVADYTTTIQGFDITNHHEV
ALTSLKVIKVWNDKDDYYHKRPKEITILLKADGKVIREHQMTPDQQGKWEYTFDQL
PVYQTGKKISYSIEEKQVAGYQAPVYEVDEGLKQVTVTNTLNPSYKLPDTGGQGVK
WYLLIGGGFIIVAILVLISLYQKHKRHNMSKP

657

The antigens of the present antigenic compositions may comprise the entire amino acid sequence of said protein or may comprise a fragment or analog thereof. Suitable fragments are N-terminal fragments of EAG and FNZ. An antigen derived from SFS may be comprised of a C-terminal part of SFS and an antigen derived from SEC may be comprised of a collagen-binding part of SEC.

A preferred antigenic composition of the present invention contains all the afore-said antigens EAG, FNZ, SFS, and SEC. Preferably, an N-terminal part of each of EAG and FNZ designated FNZN and EAG4B, respectively, a C-terminal part of SFS designated SFSC1 and a collagen-binding part of SEC designated SEC 2.16 are used in said composition.

From the above, it is evident that the present antigens that are derived from proteins of *Streptococcus equi* may comprise the entire protein, a fragment of said protein or an analog of said protein. Thus, the present invention is not limited to the fragments of proteins that are specifically disclosed herein.

A further embodiment of the present invention is concerned with a vaccine composition for protecting non-human mammals against infection of *Streptococcus equi,* which comprises an antigenic composition as disclosed above as immunizing component, and a pharmaceutically acceptable carrier.

Suitably, the present vaccine composition comprises an antigenic composition that contains all the afore-said antigens as immunizing component. Optionally, one or more of these antigens are comprised of analogs of said proteins or fragments thereof, e.g. N-terminal or C-terminal fragments.

The vaccine composition may comprise further components, such as an adjuvant. Suitably, said adjuvant stimulates systemic or mucosal immunity. Such adjuvants are well known in the art.

According to a suitable embodiment, the vaccine composition is a vaccine that protects susceptible mammals, suitably horses, against strangles caused by *Streptococcus equi* subsp. *equi*.

The vaccine composition of the present invention is provided in a physiologically administrable form. Suitably, it is administrable by subcutaneous or intranasal inoculation. Suitably, the vaccine composition of the present invention stimulates serum, mucosal and/or bronchial lavage antibody responses directed to *Streptococcus equi* antigens in mammals susceptible to *Streptococcus equi*, suitably horses.

The present invention is also related to a method for producing an antigen to be used in an antigenic composition of the present invention, which method comprises

(a) providing a DNA fragment encoding said antigen and introducing said fragment into an expression vector;

(b) introducing said vector, which contains said DNA fragment, into a compatible host cell;

(c) culturing said host cell provided in step (b) under conditions required for expression of the product encoded by said DNA fragment; and

(d) isolating the expressed product from the cultured host cell.

Preferably, said method further comprises a step (e) wherein the isolated product from step (d) is purified, e.g. by affinity chromatography or other chromatographic methods known in the art.

A further embodiment of the present invention is concerned with a method for preparation of a vaccine of the present invention, which vaccine contains as immunizing component an antigenic composition as disclosed above, said method comprising mixing said antigenic composition and a pharmaceutically acceptable carrier.

The present invention is also related to a method for the production of an antiserum, said method comprising administering an antigenic preparation of the present invention to an animal host to produce antibodies in said animal host and recovering antiserum containing said antibodies produced in said animal host.

Moreover, the present invention is concerned with a method of prophylactic or therapeutic treatment of *S. equi* infection in non-human mammals, suitably horses, comprising

administering to said mammal an immunologically effective amount of a vaccine or an antiserum of the present invention.

A suitable embodiment of the present invention is concerned with a method for protecting horses against *Streptococcus equi* infection, which comprises inoculating a horse subcutaneously or intranasally or both subcutaneously and intranasally with a vaccine of the present invention to induce an immune response against *Streptococcus equi* in said horse. Suitably, an immune response in the form of IgG and IgA antibodies in the nasopharyngeal mucus is thereby induced in said horse.

The present invention describes the composition of a vaccine comprising one or several antigen components which have been prepared according to the present method using *E. coli* as host cells. The source of these antigens might also be the native bacteria, if methods are developed for expression and purification thereof. Alternatively, the antigens of the present invention can also be produced according to methods that are based on fusion strategies where various parts of the respective antigen are recombined resulting in a fusion protein consisting of parts from different antigens. This fusion strategy could also be suitable for introducing immune reactive part(s), e.g. T-cell epitopes or attenuated toxins (or parts thereof), thereby introducing other features suitable for optimizing the antigen presentation or localization.

EXPERIMENTAL PART

Example 1. Preparation of antigens derived from EAG

A gene similar to the afore-mentioned *zag* gene (the GeneBank accession number for the nucleotide sequence of *zag* is U2582) from *S. equi* subsp. *zooepidemicus* but present in subsp. *equi* has been described in Lindmark et al (1999) (Ref. 11) and Lindmark (1999) (Ref. 13). This gene that is present in subsp. *equi* and encodes the afore-mentioned protein EAG is hereafter called *eag*. To clone and express a part of this gene encoding an N-terminal fragment (EAG4B) of EAG in *E. coli*, the following work was done.

Construction

The primers

OZAG43B: 5′-TTT TCT CGA GCT ACG GTA GAG CTG ATA AAA TCT C-3′
(SEQ. ID. NO: 5) and
OZAG15: 5′-TCA GCC ATG GCT CTA GAT GCT ACA ACG GTG TT-3′
(SEQ. ID. NO: 6)

were used to PCR-amplify a DNA-fragment corresponding to amino acid residues 34-262 in protein EAG using *S. equi* subspecies *equi* 1866 chromosomal DNA as a template. The PCR-

product was digested with *Nco*I and *Xho*I and ligated into the pTYB4-vector obtained from New England Laboratories (NEB Inc) digested with the same enzymes. One µl of the ligation mixture was transformed into *E. coli* ER2566. Correct transformants were identified by colony screening using horse radish peroxidase-labeled human serum albumin. The clone chosen for further work was sequenced from both directions to verify the correct insertion into the vector and the presence of a stop codon between EAG and the intein-affinity tag.

HSA-column

50 mg human serum albumin (HSA, Sigma) were immobilized on a 5 ml HiTrap NHS-activated column (Pharmacia Biotech) according to the manufacturer's instructions. Based on the absorbance at 280 nm, 65% of the HSA was estimated to be immobilized on the column.

Expression and purification – general

16 ml of an over night culture of EAG4B in *E. coli* ER2566 were inoculated into 2 liters of LB-medium supplemented with 50 µg ampicillin per ml. The culture was incubated at 37°C until an OD_{600} in the range of 0.5-0.6 was reached. Thereafter, IPTG was added to a final concentration of 0.5 mM and the culture was incubated at 25°C over night.

The culture was harvested and the pellet was washed once in 50 ml PBS-D before it was resuspended in 40 ml of 20 mM Na phosphate, pH 7.0 (binding buffer). The resuspension was divided into two tubes and 10 mg lysozyme were added to each tube. After 2 hrs. of incubation at 37°C, the tubes were frozen at –20°C over night.

The lysate was sonicated to fragmentize the DNA, centrifuged and the supernatant was filtered through a 0.45 µm filter.

The column was washed with 30 ml binding buffer before the sample (corresponding to 1 liter of the over night culture) was applied onto the column. Thereafter, the column was washed with 50 ml binding buffer and the A_{280} value was confirmed to be below 0.010. Bound protein was eluted in 0.1 M glycine-HCl, pH 3.0. The first 4.5 ml were not collected while the next 10 ml were collected as 1 ml fractions, which were neutralized by addition of 100 µl 1 M Tris-buffer, pH 8.0. Then, the column was regenerated by addition of binding buffer and stored at + 4°C in the presence of sodium azide.

The A_{280} value was determined and the fractions containing protein, normally fractions 1 to 5 or 6 were pooled and dialyzed 3 times against 4 liters of PBS-D. Thereafter, the protein was concentrated by extracting water from the dialysis tubing with PEG 30 000.

From an over night culture of 2 liters, this procedure yielded 5-6 mg of an N-terminal fraction of the EAG protein, designated EAG4B and comprising the amino acid sequence recited above as SEQ. ID. NO: 1. In this sequence, the first amino acid presented in bold originates from the vector.

The nucleotide sequence of the fragment encoding EAG4B where the coding sequence starts from nucleotide 38(A) and ends at nucleotide 577(C) is shown below (SEQ. ID. NO: 7).

SEQ.ID. NO: 7:

```
                10         20         30         40         50
                 |          |          |          |          |
     1 AAATAATTTTGTTTAACTTTAAGAAGGAGATATAACCATGGCTCTAGATG
    51 CTACAACGGTGTTAGAGCCTACAACAGCCTTCATTAGAGAAGCTGTTAGG
   101 GAAATCAATCAGCTGAGTGATGACTACGCTGACAATCAAGAGCTTCAGGC
   151 TGTTCTTGCTAATGCTGGAGTTGAGGCACTTGCTGCAGATACTGTTGATC
   201 AGGCTAAAGCAGCTCTTGACAAAGCAAAGGCAGCTGTTGCTGGTGTTCAG
   251 CTTGATGAAGCAAGACGTGAGGCTTACAGAACAATCAATGCCTTAAGTGA
   301 TCAGCACAAAAGCGATCAAAAGGTTCAGCTAGCTCTAGTTGCTGCAGCAG
   351 CTAAGGTGGCAGATGCTGCTTCAGTTGATCAAGTGAATGCAGCCATTAAT
   401 GATGCTCATACAGCTATTGCGGACATTACAGGAGCAGCCTTGTTGGAGGC
   451 TAAAGAAGCTGCTATCAATGAACTAAAGCAGTATGGCATTAGTGATTACT
   501 ATGTGACCTTAATCAACAAAGCCAAAACTGTTGAAGGTGTCAATGCGCTT
   551 AAGGCAAAGATTTTATCAGCTCTACCGTAGCTCGAGCCCGGGTGCTTTGC
```

Example 2. Preparation of antigens derived from SFS

The SFS protein from *S. equi* subsp. *equi* has previously been described by Lindmark and Guss (1999) (Ref. 12) and in WO 00/37496. The GeneBank accession number for the nucleotide sequence of the *sfs* gene is AF 136451.

A C-terminal fragment of this protein was produced as follows:

The 3′ end of the *sfs* gene was PCR amplified using the Taq DNA polymerase (Amersham) and chromosomal DNA from *S. equi* strain 1866 as template and the synthetic oligonucleotides

OSFS25: 5′-GGTCCCATGGCAACTCCGAATTTAGAAGGA-3′ (SEQ. ID. NO: 8) and

OSFS23: 5′-CAGACTCGAGGTCGGGATTGTAAGAATAG-3′ (SEQ. ID. NO: 9) as primers. The PCR procedure was performed in 100 µl under standard conditions as regards buffer, template and primer concentration. The PCR cycles were performed under the following conditions: 1 min. of denaturation at 94°C, 30 sec. of annealing at 40°C, and 2 min. of extension at 72°C, which were repeated as 25 cycles. After amplification, the PCR product

was purified using phenol extractions, chloroform extractions and EtOH precipitation. The purified DNA was cleaved with the restriction enzymes *Nco*I and *Xho*I after which the DNA was purified as described above.

The DNA obtained above was ligated into the plasmid vector pTYB4 [(New England Biolabs, Beverly, MA, USA (NEB Inc.)], which previously had been digested with the same restriction enzymes and treated with alkaline phosphatase. After ligation (using the ReadyToGo ligation kit, Amersham), the DNA sample was electrotransformed into the *E. coli* strain ER2566 and spread on LAA-plates (Luria Bertani agar plates supplemented with ampicillin, final conc. 50 μg/ml). After incubation over night at 37°C, clones harboring plasmids with inserts were isolated and the presence of the correct insert was verified by DNA sequencing. One of the clones thereby obtained, called SFSC1, was chosen for production of the C-terminal part of SFS.

The vector used is a part of an *E. coli* expression and purification system called IMPACT^{T} T7 (NEB Inc.). Briefly, following the manufacturer's instructions, the clone SFSC1 was grown at 37 °C in Luria Bertani growth medium supplemented with ampicillin (final conc. 50 μg/ml). At an optical density (OD_{600}) of ~ 0.6, the growth medium was supplemented with IPTG (final conc. 0.4 mM) and the growth temperature was shifted to 20°C. After incubation over night, the cells were harvested and resuspended in a buffer [20 mM Tris-HCl (pH 8.0), 500 mM NaCl, 0.1 mM EDTA, and 0.1 % Triton X100], lysed by freezing and thawing, and after centrifugation, the supernatant was sterile filtered and applied to a chitin column.

The column was extensively washed using the same buffer as above and was subsequently treated with cleavage buffer [20 mM Tris-HCl (pH 8.0), 50 mM NaCl, 0.1 mM EDTA, and 30 mM dithiothreitol (DTT)]. The reducing conditions in the cleavage buffer induce an intein-mediated self-cleavage that releases the SFS part from the column while the intein-chitin-binding part remains bound. The eluted sample containing the product SFSC1 was dialysed against phosphate-buffered saline [PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.4 mM KH_2PO_4 (pH 7.4)] and concentrated. The amount of protein obtained was determined and the quality checked using SDS-PAGE.

After the intein part has been cleaved off from SFSC1, the purified protein has an amino acid sequence as recited below as SEQ. ID. NO: 10 except that the amino acid residues in bold are residues that correspond to the amino acid residues encoded by the pTYP4 vector, while remaining amino acid residues originate from the SFS protein.

SEQ. ID. NO: 10:

MATPNLEGNN RKNPLKGLDG ENKPKEDLDG KGLSGENDES PKLKDEHPYN HGRRDGYRVG YEDGYGGKKH KGDYPKRFDE SSPKEYNDYS QGYNDNYGNG YLDGLADRGG KRGYGYSYNP DLEPG.

Example 3. Preparation of antigens derived from FNZ

Protein FNZ from *S. equi* subsp. *zooepidemicus* has previously been described by Lindmark et al (1996) (Ref. 9), Lindmark (1999) (Ref. 13), Lindmark et al (1999) (Ref. 11) and Lindmark et al (2001) (Ref. 14). The EMBL accession number for the nucleotide sequences of the *fnz* gene is X99995. A similar truncated protein called FNE is also expressed by strains of *S. equi* subsp. *equi* [Lindmark et al (2001), Ref. 14]. The GeneBank accession number for the nucleotide sequence of the *fne* gene is AF360373. The construction of a clone called pT2fnzN encoding the N-terminal part of FNZ has previously been described by Lindmark et al 2001 (Ref. 14). Briefly, the clone pT2fnzN was constructed as follows using PCR amplification with the forward primer

OFNZ1: 5′-ACCATGGCTAGCGCAGAGCAGCTTTATTATGGGT-3′ (SEQ. ID. NO: 11),

and the reverse primer

OFNZ2: 5′- ATACCCGGGATATCCTTCGGTACTACCATAGT-3′ (SEQ. ID. NO: 12).

Chromosomal DNA from subsp. *zooepidemicus* strain ZV was used as the template and the 5′end of the *fnz* gene was amplified. The PCR fragment obtained was cleaved with restriction endonucleases *Nhe*I and *Sma*I, followed by ligation into the corresponding restriction endonuclease sites in the expression vector pTYB2 (NEB). The ligated DNA was electrotransformed into *E. coli* strain ER2566. Plasmids harboring inserts were isolated from transformants and verified by DNA sequencing. One such clone is designated pT2fnzN. The production in *E .coli* and purification of the N-terminal part of FNZ, called FNZN, were performed as described above for protein SFSC1 and these steps are also described in Lindmark et al (2001) (Ref. 14).

The amino acid sequence of FNZN comprises then amino acid residues 32-337 of the amino acid sequence of the protein FNZ recited above as SEQ. ID. NO: 2. The corresponding amino acid sequence of FNZN is shown below as SEQ. ID. NO: 13.

SEQ. ID. NO: 13

M A S A E Q L Y Y G W N D G T R Q S S P
Y F L Y V S P K N A P K R E L K D E Y V
V Y C F N K K L Y W P D Q W E S I Y S N
F N D I R S P Y N D L P V Y E K K L G Y
D G I F K Q Y A P D Y K K D I S D I A S

A L V A V L S N G Y P T N K S Q L S T S
Y H L N N D S S R K V T Q L A I W Y F S
D S L T K E Y L K D T G G Y N L N D M E
K K A L D F L I S K G E D S K L K S E Q
S N Y S L D I Y V Y Q S G G H D H M K D
Y Q N L L G S T L I P K E P L K P Q L G
G F S G H N G N G L S G L E G G S S G S
Q E T N E D G K K G L I G F H G G L S G
S E G K R D P L P G L K G E A G A P D T
P Q K P N D P L Q G L E G G N S P I V E
Q N Y G S T E G Y G

In the previously recited amino acid sequence (SEQ. ID. NO: 2) of protein FNZ, amino acids shown in bold originate from the protein FNZ in S. *equi* subspecies *zooepidemicus*, while those shown in normal type are derived from the expression vector construct. In SEQ. ID. NO:13, the first three and the last amino acid residues originate from the vector.

Example 4. Preparation of antigens derived from the protein SEC

The genome of *S. equi* is accessible (www.sanger.ac.uk) for computer analysis. By using earlier published sequences of virulence factors or potential virulence factors from pathogenic streptococci and staphylococci, it is possible to screen the genome of *S. equi* for the presence of similar genes. By using the soft ware program BLAST and searching for open reading frames encoding a protein similar to the collagen-binding protein CNA of *Staphylococcus aureus* (WO 92/07002), it was possible to identify a hypothetical gene of 1971 nucleotides in the *S. equi* genome encoding a protein of 657 amino acids. (Ref. 25, Lannergård et al. (2003)).This protein, hereafter termed protein SEC (SEQ. ID. NO: 4), encodes an N-terminal signal sequence (amino acid sequence 1-26) followed by a region, which displays collagen-binding. The C-terminal part of protein SEC contains all typical features of cell surface proteins from streptococci, like putative wall anchoring and membrane spanning regions, as well as a motif corresponding to the LPDTG motif (SEQ. ID. NO: 14) of the protein SEC.

The nucleotide sequence of the *sec* gene (later renamed to *cne*) encoding the protein SEC (the accession no. of the *cne* is AY193773). is shown below as SEQ. ID. NO: 15.

SEQ. ID. NO: 15:

1
TTGAAACAACTGACAAAGATCGTTAGTGTGGTCTTGTTGCTGGTCTTTACCCTTAG
TGCTAGCCTGCACAAGGTTCGGGCAACTAATCTTAGTGACAACATCACATCATTG
ACGGTTGCTTCTTCATCACTCCGAGATGGAGAGAGAACGACGGTAAAGGTTGCGT
TTGATGACAAAAAACAGAAAATCAAGGCAGGGGATACGATAGAGGTCACCTGGC
CTACAAGTGGTAATGTCTACATTCAGGGCTTTAATAAAACCATACCGCTTAATAT
TAGAGGGGTAGATGTTGGTACCTTGGAGGTCACGCTAGACAAGGCTGTTTTCACA
TTCAATCAAAATATTGAAACAATGCATGATGTCTCTGGTTGGGGAGAGTTTGATA
TTACTGTTAGAAATGTGACACAAACCACCGCTGAAACATCAGGAACGACCACAG
TAAAGGTAGGCAATCGCACTGCTACTATCACTGTTACTAAGCCTGAGGCAGGCAC
TGGTACCAGCTCATTTTATTATAAGACTGGTGATATGCAGCCCAATGATACTGAG
CGTGTGAGATGGTTCCTGCTGATTAACAACAACAAGGAATGGGTGGCCAATACTG
TTACAGTCGAAGACGATATTCAAGGTGGTCAAACCTTGGATATGAGCAGCTTTGA
CATCACCGTATCTGGTTATCGTAACGAGCGCTTCGTTGGGGAAAACGCTCTGACA
GAGTTTCATACAACATTTCCAAATTCTGTCATTACGGCAACAGATAATCACATTA
GTGTGCGGTTAGATCAATATGATGCCTCACAAAACACTGTCAACATTGCTTATAA
GACAAAGATAACGGACTTTGACCAAAAAGAATTTGCCAACAACAGTAAAATCTG
GTACCAGATTTTATACAAGGATCAGGTATCGGGTCAAGAGTCAAACCACCAAGTA
GCCAATATCAATGCTAACGGCGGGGTTGATGGCAGTCGCTATACCAGCTTTACTG
TCAAGAAAATTTGGAATGACAAGGAAAATCAAGACGGTAAGCGTCCAAAGACTA
TTACTGTTCAGCTTTACGCCAATGATCAGAAAGTTAATGATAAGACCATTGAATT
GAGTGATACTAATAGCTGGCAAGCAAGTTTTGGTAAGCTGGATAAGTATGACAGT
CAGAACCAAAAAATTACCTACAGTGTCAAGGAAGTGATGGTTCCTGTTGGCTACC
AATCGCAGGTTGAGGGGGATAGTGGAGTAGGATTTACCATTACCAACACCTATAC
ACCAGAGGTCATTAGCATTACCGGTCAAAAAACTTGGGACGACAGGGAAAACCA
AGACGGTAAACGTCCTAAGGAGATTACGGTTCGTTTATTGGCAAATGACGCTGCA
ACTGACAAGGTAGCAACTGCTTCAGAGCAAACCGGCTGGAAGTATACATTTACCA
ATCTACCGAAATACAAAGATGGTAAACAGATCACCTACACGATCCAAGAGGACC
CTGTGGCAGATTACACCACAACCATTCAGGGATTTGATATTACCAATCATCATGA
GGTAGCCTTGACCAGCCTAAAGGTCATCAAGGTTTGGAATGATAAGGACGATTAT
TACCATAAACGTCCCAAGGAGATTACCATTTTGCTAAAGGCAGATGGCAAGGTGA
TTCGTGAACATCAGATGACACCGGATCAGCAAGGAAAATGGGAATACACCTTTG
ACCAGCTGCCGGTCTATCAGACAGGCAAGAAAATCAGCTACAGCATTGAGGAAA
AACAGGTTGCTGGCTATCAAGCCCCTGTCTATGAGGTTGATGAAGGCTTGAAGCA
GGTCACTGTAACCAACACCCTTAACCCAAGCTACAAGCTGCCTGACACCGGAGGA
CAAGGAGTGAAATGGTACCTGTTAATCGGTGGCGGTTTTATCATCGTCGCAATCC
TTGTACTGATCAGCCTTTATCAAAAACACAAGCGCCATAACATGTCAAAACCA
1971

Construction of recombinant protein SEC expressing collagen-binding activity

To express and purify the collagen-binding activity of protein SEC, two different constructions were made. The first construct, encoding the major part of protein SEC was made as follows. The primers

OSEC1:5: 5′-CATGCCATGGCAACTAATCTTAGTGACAACAT-3′ (SEQ. ID. NO: 16)

and

OSEC3:3: 5′-CCGCTCGAGCTTGTAGCTTGGGTTAAGGGTGT -3′ (SEQ. ID. NO: 17)

were used to PCR-amplify a DNA-fragment corresponding to a sequence from amino acid no.

27 to amino acid no. 615 in protein SEC (SEQ. ID. NO: 4) using *S. equi* subspecies *equi* 1866 chromosomal DNA as a template.

The second construct encoding the N-terminal part of protein SEC was made as follows. The primers OSEC1:5 and

OSEC2:3: 5'-CCGCTCGAGAAAGCTGGTATAGCGACTGCCAT-3' (SEQ. ID. NO: 18) were used to PCR-amplify a DNA-fragment corresponding to a sequence from amino acid no. 27 to amino acid no. 328 in protein SEC (SEQ. ID. NO: 4) using *S. equi* subspecies *equi* 1866 chromosomal DNA as a template.

Both PCR amplifications were performed using ReadyToGo™ PCR beads (Amersham Pharmacia Biotech Inc) and the PCR apparatus MiniCycler™ (MJ Research, Inc, MA, USA) using a program comprising: step 1: 95°C, 1 min; step 2: 95°C, 30 sec.; step 3: 46°C, 15 sec.; and step 4: 72°C, 2 min; repeated as 25 cycles from step 2 to step 4. After PCR amplification, the respective PCR-products were purified and digested with restriction endonucleases *Nco*I and *Xho*I and ligated into the pTYB4-vector (NEB) digested previously with the same enzymes. One µl of the respective ligation mixture was transformed into *E. coli* ER2566. After IPTG induction, *E. coli* clones expressing collagen-binding were identified by colony screening using ^{125}I-labeled collagen (collagen S, type I obtained from Boeringher Mannheim). Several clones of both types of constructions expressing collagen-binding were identified and further characterized. One of these clones called pSEC 2.16 harboring the PCR-fragment and originating from the PCR using OSEC 1:5 and 3:3 was chosen after DNA sequencing of the insert for production of a recombinant collagen-binding protein called protein SEC 2.16 and comprising amino acid residues 27 - 616 of SEQ. ID. NO: 4. The nucleotide sequence of this insert encoding the recombinant collagen-binding protein SEC2.16 is shown below as SEQ. ID. NO: 19. The nucleotides shown in bold represent nucleotides originating from the vector.

SEQ. ID. NO: 19: Insert of pSEC2.16

ATGGCAACTAATCTTAGTGACAACATCACATCATTGACGGTTGCTTCTTCATCACTCCGAGATGGA
GAGAGAACGACGGTAAAGGTTGCGTTTGATGACAAAAAACAGAAAATCAAGGCAGGGGATACGA
TAGAGGTCACCTGGCCTACAAGTGGTAATGTCTACATTCAGGGCTTTAATAAAACCATACCGCTTA
ATATTAGAGGGGTAGATGTTGGTACCTTGGAGGTCACGCTAGACAAGGCTGTTTTCACATTCAAT
CAAAATATTGAAACAATGCATGATGTCTCTGGTTGGGGAGAGTTTGATATTACTGTTAGAAATGTG
ACACAAACCACCGCTGAAACATCAGGAACGACCACAGTAAAGGTAGGCAATCGCACTGCTACTAT
CACTGTTACTAAGCCTGAGGCAGGCACTGGTACCAGCTCATTTTATTATAAGACTGGTGATATTCA
GCCCAATGATACTGAGCGTGTGAGATGGTTCCTGCTGATTAACAACAACAAGGAATGGGTGGCC
AATACTGTTACAGTCGAAGACGATATTCAAGGTGGTCAAACCTTGGATATGAGCAGCTTTGACATC
ACCGTATCTGGTTATCGTAACGAGCGCTTCGTTGGGGAAAACGCTCTGACAGAGTTTCATACAAC
ATTTCCAAATTCTGTCATTACGGCAACAGATAATCACATTAGTGTGCGGTTAGATCAATATGATGC
CTCACAAAACACTGTCAACATTGCTTATAAGACAAAGATAACGGACTTTGACCAAAAAGAATTTGC

CAACAACAGTAAAATCTGGTACCAGATTTTATACAAGGATCAGGTATCGGGTCAAGAGTCAAACC
ACCAAGTAGCCAATATCAATGCTAACGGCGGGGTTGATGGCAGTCGCTATACCAGCTTTACTGTC
AAGAAAATTTGGAATGACAAGGAAAATCAAGACGGTAAGCGTCCAAAGACTATTACTGTTCAGCTT
TACGCCAATGATCAGAAAGTTAATGATAAGACCATTGAATTGAGTGATACTAATAGCTGGCAAGCA
AGTTTTGGTAAGCTGGATAAGTATGACAGTCAGAACCAAAAAATTACCTACAGTGTCAAGGAAGT
GATGGTTCCTGTTGGCTACCAATCGCAGGTTGAGGGGGATAGTGGAGTAGGATTTACCATTACCA
ACACCTATACACCAGAGGTCATTAGCATTACCGGTCAAAAAACTTGGGACGACAGGGAAAACCAA
GACGGTAAACGTCCTAAGGAGATTACGGTTCGTTTATTGGCAAATGACGCTGCAACTGACAAGGT
AGCAACTGCTTCAGAGCAAACCGGCTGGAAGTATACATTTACCAATCTACCGAAATACAAAGATG
GTAAACAGATCACCTACACGATCCAAGAGGACCCTGTGGCAGATTACACCACAACCATTCAGGGA
TTTGATATTACCAATCATCATGAGGTAGCCTTGACCAGCCTAAAGGTCATCAAGGTTTGGAATGAT
AAGGACGATTATTACCATAAACGTCCCAAGGAGATTACCATTTTGCTAAAGGCAGATGGCAAGGTG
ATTCGTGAACATCAGATGACACCGGATCAGCAAGGAAAATGGGAATACACCTTTGACCAGCTGC
CGGTCTATCAGGCAGGCAAGAAAATCAGCTACAGCATTGAGGAAAAACAGGTTGCTGGCTATCAA
GCCCCTGTCTATGAGGTTGATGAAGGCTTGAAGCAGGTCACTGTAACCAACACCCTTAACCCAAG
CTACAAG**CTCGAGCCCGGG**

The amino acid sequence of the recombinant collagen-binding protein SEC2.16 encoded by the insert of pSEC2.16 is shown below as SEQ. ID. NO: 20. The amino acids shown in bold represent amino acids originating from the vector.

SEQ. ID. NO: 20: Protein SEC2.16

MATNLSDNITSLTVASSSLRDGERTTVKVAFDDKKQKIKAGDTIEVTWPTSGNVYIQGFNKTIPLNIRGV
DVGTLEVTLDKAVFTFNQNIETMHDVSGWGEFDITVRNVTQTTAETSGTTTVKVGNRTATITVTKPEA
GTGTSSFYYKTGDIQPNDTERVRWFLLINNNKEWVANTVTVEDDIQGGQTLDMSSFDITVSGYRNER
FVGENALTEFHTTFPNSVITATDNHISVRLDQYDASQNTVNIAYKTKITDFDQKEFANNSKIWYQILYKD
QVSGQESNHQVANINANGGVDGSRYTSFTVKKIWNDKENQDGKRPKTITVQLYANDQKVNDKTIELS
DTNSWQASFGKLDKYDSQNQKITYSVKEVMVPVGYQSQVEGDSGVGFTITNTYTPEVISITGQKTWD
DRENQDGKRPKEITVRLLANDAATDKVATASEQTGWKYTFTNLPKYKDGKQITYTIQEDPVADYTTTIQ
GFDITNHHEVALTSLKVIKVWNDKDDYYHKRPKEITILLKADGKVIREHQMTPDQQGKWEYTFDQLP
VYQAGKKISYSIEEKQVAGYQAPVYEVDEGLKQVTVTNTLNPSYK**LEPG**

The other clone obtained above that was chosen for further studies was called pSEC 1.18 and harbored the PCR-fragment originating from the PCR using OSEC 1:5 (SEQ. ID. NO. 16) and 2:3 (SEQ. ID. NO. 18). After DNA sequencing of the insert of pSEC 1.18, this clone was used for production of a recombinant collagen-binding protein called protein SEC 1.18. This protein SEC 1.18 comprises the amino acid sequence shown below as SEQ. ID. NO. 22. The corresponding nucleotide sequence is shown below as SEQ. ID. NO. 21. In this nucleotide sequence, the nucleotides shown in bold represent nucleotides originating from the vector.

SEQ. ID. NO. 21: Insert of pSEC1.18

ATGGCAACTAATCTTAGTGACAACATCACATCATTGACGGTTGCTTCTTCATCACTCCGAGATGGA
GAGAGAACGACGGTAAAGGTTGCGTTTGATGACAAAAAACAGAAAATCAAGGCAGGGGATACGA
TAGAGGTCACCTGGCCTACAAGTGGTAATGTCTACATTCAGGGCTTTAATAAAACCATACCGCTTA
ATATTAGAGGGGTAGATGTTGGTACCTTGGAGGTCACGCTAGACAAGGCTGTTTTCACATTCAAT
CAAAATATTGAAACAATGCATGATGTCTCTGGTTGGGGAGAGTTTGATATTACTGTTAGAAATGTG

ACACAAACCACCGCTGAAACATCAGGAACGACCACAGTAAAGGTAGGCAATCGCACTGCTACTAT
CACTGTTACTAAGCCTGAGGCAGGCACTGGTACCAGCTCATTTTATTATAAGACTGGTGATATGC
AGCCCAATGATACTGAGCGTGTGAGATGGTTCCTGCTGATTAACAACAACAAGGAATGGGTGGC
CAATACTGTTACAGTCGAAGACGATATTCAAGGTGGTCAAACCTTGGATATGAGCAGCTTTGACAT
CACCGTATCTGGTTATCGTAACGAGCGCTTCGTTGGGGAAAACGCTCTGACAGAGTTTCATACAA
CATTTCCAAATTCTGTCATTACGGCAACAGATAATCACATTAGTGTGCGGTTAGATCAATATGATG
CCTCACAAAACACTGTCAACATTGCTTATAAGACAAAGATAACGGACTTTGACCAAAAAGAATTTG
CCAACAACAGTAAAATCTGGTACCAGATTTTATACAAGGATCAGGTATCGGGTCAAGAGTCAAAC
CACCAAGTAGCCAATATCAATGCTAACGGCGGGGTTGATGGCAGTCGCTATACCAGCTTT**CTCGA**
GCCCGGG

The amino acid sequence of the recombinant collagen-binding protein SEC1.18 encoded by the insert of pSEC1.18 is shown below as SEQ. ID. NO: 22. The amino acids shown in bold represent amino acids originating from the vector.

SEQ. ID. NO: 22: Protein SEC1.18.

MATNLSDNITSLTVASSSLRDGERTTVKVAFDDKKQKIKAGDTIEVTWPTSGNVYIQGFNKTIPLNIRGV
DVGTLEVTLDKAVFTFNQNIETMHDVSGWGEFDITVRNVTQTTAETSGTTTVKVGNRTATITVTKPEA
GTGTSSFYYKTGDMQPNDTERVRWFLLINNNKEWVANTVTVEDDIQGGQTLDMSSFDITVSGYRNER
FVGENALTEFHTTFPNSVITATDNHISVRLDQYDASQNTVNIAYKTKITDFDQKEFANNSKIWYQILYKD
QVSGQESNHQVANINANGGVDGSRYTSF**LEPG**

Production of protein SEC 1.18 and protein SEC 2.16

Since the vector used to construct the pSEC1.18 and pSEC 2.16 is a part of the IMPACT-system (NEB), the expression and purification of protein SEC1.18 and protein SEC2.16 were performed as described for protein SFSC1 and protein FNZN. After purification, protein SEC 1.18 and protein SEC 2.16 were analysed by SDS-PAGE using the Phast-System (Amersham Pharmacia) and 8-25% gradient gels under reducing conditions (SDS buffer strips, boiling the samples in a sample buffer containing SDS and β-mercapto-ethanol before applying the samples on the gels). The results show that the purified protein SEC 1.18 and protein SEC 2.16, approx. had relative molecular weights corresponding to their calculated molecular weights.

Collagen-binding activity.

The purified proteins SEC 1.18 and SEC 1.16 were also analysed for their ability to bind to collagen. First, the proteins were run on an SDS-PAGE 8-25% gel (Amersham Pharmacia) under reducing conditions. After the electrophoresis was completed, the proteins were transferred (by diffusion) to a nitro-cellulose membrane. The membrane was blocked in a solution (PBS-T) containing PBS supplemented with TWEEN 20 (final concentration 0.5%) and casein 0.5% (final conc.) for 1 hr. at room temperature (RT). After washing with PBS-T (casein omitted), the membrane was transferred to a solution of PBS-T containing ^{125}I-labeled

collagen. After 4 hrs. of incubation at RT under gentle agitation, the membrane was extensively washed using PBS-T (casein omitted) and subjected to autoradiography using Biomax MS (Kodak) film. The results showed that in contrast to unrelated proteins used as control (size marker proteins), both protein SEC 1.18 and protein SEC 2.16 bound collagen.

Occurrence of the *sec* gene in S. equi

Using chromosomal DNA from various strains of S. *equi* subspecies *equi* and subspecies *zooepidemicus* as template and the primer combinations OSEC1:5 (SEQ. ID. NO: 16) and OSEC 2:3 (SEQ. ID. NO: 18) or OSEC1:5 (SEQ. ID. NO: 16) and OSEC 3:3 (SEQ. ID. NO: 17), the presence of the *sec* gene was investigated. The PCR conditions applied were the same as described above in the section "Construction of recombinant protein SEC expressing collagen-binding activity". The result showed that the *sec* gene was present in all tested strains in both subspecies. This is an important finding, since a vaccine containing an antigen, which is present in both subspecies, gives the possibility to broaden the application of the invention resulting in a vaccine active against infections of both subspecies.

Further search for vaccine components.

The genome of *S. equi* is accessible (www.sanger.ac.uk) for computer analysis. As for protein SEC, it is possible to further screen the genome of *S. equi* for genes encoding earlier published sequences of virulence factors or potential virulence factors from pathogenic streptococci and staphylococci. Use of the soft ware program BLAST resulted in the identification in *S. equi* of additional genes encoding potential virulence factors similar to *Streptococcus pyogenes*: C5a peptidase (gi:14195215, sp, P58099,SCA2 STRPY), SclB (emb: CAC33776.1), collagen-like protein similar to (ref: NP358996.1), speK (ref: NP 438166.1), exotoxin I (gi:16923748, gb:AAL31571.1, AF438524.1), streptodornase (emb:CAA59264.1), a DNA entry nuclease (ref: NP 346391.1), trypsin resistant surface T6 protein (sp:P18481, TEE6 STRPY), M and M-like proteins, other fibronectin-binding proteins and other.

Furthermore, the computer analyses revealed several hypothetical proteins displaying a structure which is found in cell wall-associated proteins in Gram-positive cocci (Schneewind et al 1995, Ref. 15). At the N-terminal end of these proteins, a signal sequence is found and at the C-terminal end, a motif LPXTG corresponding to the LPDTG motif (SEQ. ID. NO: 14) of the SEC protein is present, which is followed by a stretch of hydrophobic amino acids ending with a few charged amino acids constituting the membrane-spanning region. These proteins should also be considered as potential targets to be included in an efficient vaccine against *S. equi* infections.

Based on considerations as discussed above, two proteins have been prepared and evaluated as disclosed in the following Examples 5 and 6.

Example 5. Preparation of protein SclC and protein SCLC1

The amino acid sequence of *Streptpcoccus pyogenes* protein SclB was used to screen the genome of *S. equi* (www.sanger.ac.uk) using the software program BLAST. As a result of this screening, an open reading frame was identified, which encodes a protein called SclC, which is similar to SclB. The protein SclC comprises an amino acid sequence as shown as SEQ. ID. NO: 23 below.

SEQ. ID. NO: 23:

1

M T N K T K R T G L V R K Y G A C S A A I A L A A L A S L G A G K A V K A D
Q P A A L K Y P E P R D Y F L H T R E G D V I Y D E D I K R Y F E D L E A Y
L T A R L G G I D K K V E E A A Q K P G I P G P T G P Q G P K G D K G D P G
A P G E R G P A G P K G D T G E A G P R G E Q G P A G Q A G E R G P K G D
P G A P G P K G E K G D T G A V G P K G E K G D T G A T G P K G D K G E R
G E K G E Q G Q R G E K G E Q G Q R G E K G E Q K P K G D Q G K D T K P
S A P K A P E K A P A P K A P K A S E Q S S N P K A P A P K S A P S K S A A
P T G Q K A A L P A T G E I N H P F F T L A A L S V I A S V G V L T L K G K
K D

302

The nucleotide sequence of the gene sclC encoding protein SclC is shown below as SEQ. ID. NO: 24.

SEQ. ID. NO: 24:

1

atgacaaacaaaacaaagcgtacaggattggtacgcaagtacggtgcctgctcagcagctatcgccttagcagctcttgcaagcctgg
gagcaggtaaagcagtaaaggcagaccagccagcagcactaaaatatccagaacctagagactattttcttcatactcgtgaaggtgat
gttatttatgatgaggatataaaaagatattttgaggatttagaagcctatttaacagctagacttggtgggattgataaaaaagtagaagaa
gctgcccaaaagccaggtattccaggtcctactggccctcaaggtcctaagggagacaaaggagatccaggtgcccctggtgagcgc
ggtccagctggaccaaagggcgatacgggcgaagccggaccaagaggtgagcaaggcccagccggacaagctggagaacgtgg
accaaaaggagatccaggtgctccaggtcctaaaggtgaaaagggtgatactggtgcagttggtcctaaaggtgaaaaaggtgatacc

ggagcaaccggaccaaagggagacaagggcgaacgcggtgaaaaaggcgagcaaggccaacgtggcgaaaaaggcgagcaa
ggccaacgcggtgaaaaaggcgagcaaaaaccaaagggtgatcaaggaaaagatacaaaaccatcagctccaaaagcacctgaaa
aggctcctgcaccaaaagctccaaaggcttcagagcagtcatctaatcctaaagcaccagctcctaagtcagcaccaagcaaatcagc
ggcaccaacaggtcaaaaagcagccctaccagcaacaggggaaatcaaccacccattcttcacccttgcagctcttagtgtcatcgcta
gcgtaggcgtcctaactctaaaaggaaaaaaagactaa

909

To express and purify the major part of the SclC protein, the following construction was made. The primers

OSCL2:5: 5′- CATGCCATGGACCAGCCAGCAGCACTAAAATAT-3′ (SEQ. ID. NO: 25)

and OSCL3:3: 5′- CCGCTCGAGGGCTGCTTTTTGACCTGTTGGT-3′ (SEQ. ID. NO: 26)

were used to PCR-amplify a DNA-fragment corresponding to amino acid 38 to amino acid 269 in protein SclC using *S. equi* subspecies *equi* 1866 chromosomal DNA as a template. PCR amplification was performed using ReadyToGo™ PCR beads (Amersham Pharmacia Biotech Inc) and the PCR apparatus MiniCycler™ (MJ Research, Inc, MA, USA) using a program of step 1: 95°C, 1 min; step 2: 95°C 30 sec.; step 3: 50°C 15 sec.; step 4: 72°C, 1 min; repeated as 24 cycles from step 2 to step 4. After PCR amplification, the respective PCR-products were purified and digested with restriction endonucleases *Nco*I and *Xho*I and ligated into the pTYB4-vector (NEB) previously digested with the same enzymes. One µl of the respective ligation mixture was transformed into *E. coli* ER2566. *E. coli* clones harboring the *sclc* fragment were identified by DNA sequencing of the inserted fragments.

Of these clones, one clone called pSclC1 and harboring the PCR-fragment originating from the PCR using OSCL2:5 (SEQ. ID. NO: 25) and OSCL3:3 (SEQ. ID. NO: 26), was chosen for production of a recombinant SclC protein called protein SCLC1. This protein SCLC1 comprises the amino acid sequence shown below as SEQ. ID. NO: 27.

SEQ. ID. NO: 27:

M D Q P A A L K Y P E P R D Y F L H T R E G D V I Y D E D I K R Y F E D L E
A Y L T A R L G G I D K K V E E A A Q K P G I P G P T G P Q G P K G D K G D
P G A P G E R G P A G P K G D T G E A G P R G E Q G P A G Q A G E R G P K
G D P G A P G P K G E K G D T G A V G P K G E K G D T G A T G P K G D K G
E R G E K G E Q G Q R G E K G E Q G Q R G E K G E Q K P K G D Q G K D T
K P S A P K A P E K A P A P K A P K A S E Q S S N P K A P A P K S A P S K S
A A P T G Q K A A **L E P G**

In the peptide sequence of the insert of pSclC1 encoding the recombinant protein SCLC1 (SEQ. ID. NO: 27), the amino acids shown in bold represent amino acids originating from the vector.

Production of protein SCLC1

Since the vector used to construct the pSclC1 is a part of the IMPACT-system (NEB) the expression and purification of protein SCLC1 was performed as described for the other IMPACT-constructions (proteins SFSC1, FNZN, SEC 1.18 and SEC 2.16). After purification protein SCLC1 was analysed by SDS-PAGE using the Phast-System (Amersham Pharmacia) and 8-25% gradient gels under reducing conditions (SDS buffer strips, boiling the samples in a sample buffer containing SDS and beta-mercaptoethanol before applying the samples on the gels). The results showed that the purified protein had a relative molecular weight corresponding to its calculated molecular weight. The purified protein SCLC1 was then used to immunize mice and rabbits and was also used to screen convalescence sera from horses for measurements of antibody titers against SCLC1. This is illustrated in Example 6 below.

Example 6. Immunization of rabbits using protein SCLC1 and Western blot

Protein SCLC1 was used to immunize a rabbit. This work was done at the company Agrisera (Vännäs, Sweden). Using an ELISA format, where the SCLC1 protein was immobilized in microtiter wells, the immune serum obtained was analysed for presence of antibodies against SCLC1. The results showed that the immune sera could be diluted > 30 000 and still react with the SCLC1 protein (as compared to the pre-immune serum, which did not contain any significant level of antibodies against SCLC1).

In a Western blot analysis, the SCLC1 protein was also run on an SDS-PAGE gel using the Phast-System (Amersham Pharmacia) and 8-25% gradient gel under reducing conditions. After the electrophoresis had been completed, the SCLC1 protein was diffusion blotted to a nitrocellulose-membrane. After blocking, the membrane was divided and immune serum (dilution 1:10 000 in PBS-0.05%Tween 20) and pre-immune serum, respectively, were added and incubated for two hours at room temp. After washing in PBS-0.05%Tween 20, secondary antibodies (anti rabbit IgG, horseradish-labelled and developed in goat, Sigma) were used to detect rabbit antibodies directed against SCLC1. The result showed that the immune serum detected the immobilized SCLC1 protein efficiently, while no detection was seen using the pre-immune serum.

Occurrence of sclC gene in strains of subsp. *equi* and subsp. *zooepidemicus*

Chromosomal DNA from different isolates of both subsp. was used as template for a PCR-analysis to study the occurrence of the sclC gene. The PCR primers used were OSCL2:5 (SEQ. ID. NO: 25) and OSCL3:3 (SEQ. ID. NO: 26). The result showed that a similar gene was present in all studied strains of subsp. *equi* and in a majority of strains in subsp. *zooepidemicus*.

In the following Examples 7-10, immunization and challenge of mice were performed as described below.

Bacterial strain and growth: *Streptococcus equi* subsp. *equi* was isolated from a horse with strangles. After one passage in a mouse (inoculated intranasally and reisolated from a submandibular gland), the bacteria were stored at –80°C. To prepare an inoculum, stored bacteria were first grown on a blood agar plate, subcultured overnight at 37°C in Todd Hewitt medium containing 1% (w/v) yeast extract. Six ml of this culture were taken into 50 ml of Todd Hewitt /Yeast extract medium (BBL, Becton Dickinson) containing 10% (v/v) equine, i.e. horse, serum (heat inactivated) and incubated at 37°C for 4 hrs. Bacteria grown this way were used in animal challenge experiments.

Mice and experimental design: NMRI outbred female mice (Möllegård, Denmark) having a body weight of about 20 g, were individually marked, and kept in groups of four in each cage. Daily, they were weighed and nose impressions onto a BG plate (sheep blood and gentian violet) were taken to quantify bacterial growth. After incubation at 37°C in 5 % CO_2 for 24 hrs, the amount of haemolytic colonies was determined according to a scale of 0-3. A score of 3 means confluent growth of *Streptococcus* subsp *equi* on the whole plate and a score of 0 indicates 0-4 colonies.

Bronchoalveolar lavage (BAL) and nasal wash (NW) for determination of mucosal IgA responses: BAL was performed by infusing and withdrawing a solution consisting of 1 ml ice-cold PBS containing protease inhibitor (Complete, Roche) three times into the trachea. NW was performed by rinsing the nasal cavity with 0.5 ml ice-cold PBS containing protease inhibitor. The BAL and NW samples were kept on ice before storage at –80°C.

Statistical analysis: Differences between the groups were evaluated by using the Student *t*-test, where $p < 0.05$ was considered to be statistically significant.

Example 7: Immune response in mice subjected to infection with *Streptococcus* subsp. *equi*

Mice (n=19) were infected with *Streptococcus* subsp. *equi*. Ten µl of bacteria containing $1x10^6$ CFU were given intranasally to anaesthetized mice, viz. 5 µl in each nostril. After two weeks, the mice were sacrificed and blood samples were taken. All mice had developed IgG antibodies against FNZN, SFSC1, and EAG4B. The results from eight mice are shown in Figures 1, 2, and 3 for FNZN, SFSC1, and EAG4B, respectively. These ELISA tests were performed according to a standard procedure. Briefly, wells on microtiter plates (Costar) were coated with the respective proteins at a concentration of 10 µg/ml. Serum

dilutions from the mice samples were applied. After washing, rabbit antibodies against mice IgG conjugated with horse radish peroxidase (Dako) were added. To develop the color reaction, OPD tablets (Dako) were added according to instructions from the manufacturer.

Example 8: Immune response in mice immunized with FNZN, SFSC1, and EAG4B

Mice (n=24) were immunized subcutaneously on days 0, 7, 14, and 21. Twelve mice were given antigens (FNZN, SFSC1, and EAG4B) and adjuvant and twelve mice were given only adjutvant. The adjuvant used was EtxB provided by Dr Tim R. Hirst, University of Bristol. Each animal was given 12 µg of each antigen and 36 µg of EtxB at each occasion. On day 28, the mice were sacrificed and blood samples were taken. ELISA tests were performed as described above. All mice developed IgG antibodies against FNZN, SFSC1, and EAG4B. Samples from seven mice and a negative control are shown in Figures 4, 5, and 6 for FNZN, SFSC1, and EAG4B, respectively.

Example 9: Immune response in mice vaccinated with SEC2.16

Mice (n=5) were subcutaneously immunized on days 0, 7, 14, and 21. Each animal was given 12 µg of SEC2.16 in Freund's adjuvant. Serum samples were taken on day 28 and ELISA tests were performed as described above. In Figure 7, the production of IgG antibodies against SEC2.16 is shown.

Example 10: Subcutaneous immunization with FNZN, SFSC1, and EAG4B followed by challenge with *Streptococcus equi* subsp *equi*

Mice (n=24) were subcutaneously immunized on days 0, 7, 14, and 21. Twelve mice were given antigens (FNZN, SFSC1, and EAG4B) and the adjuvant EtxB and twelve mice were given only adjuvant. Each animal was given 12 µg of each antigen and 36 µg of EtxB at each occasion. On day 28, the mice were infected with *Streptococcus equi* subsp. *equi*. Ten µl of bacteria containing $1x10^6$ CFU were given intranasally to anaesthetized mice. Animals that lost more than 15% of weight were killed. Mice that had survived or had lost less than 15% of weight are shown in Figure 8. It is evident from Figure 8 that non-vaccinated animals had a lower survival rate than vaccinated animals. On day 3, the p-value was < 0.05. From Figure 9, it is evident that in comparison to non-vaccinated animals, fewer bacteria grew in the noses of vaccinated animals; p-values obtained on days 2, 3, and 7 are 0.03, 0.02, and 0.01, respectively.

Example 11. Intranasal immunization with FNZN, SFSC1, EAG4B and SEC2.16 followed by challenge with *Streptococcus equi* subsp. *equi*

One group of mice (n=24) was intranasally immunized on days 0, 7, 14, and 21 by administration of the antigens in 40 µl as several small doses in both nostrils. Twelve mice received the antigens FNZN, SFSC1, and EAG4B together with the adjuvant EtxB and twelve mice received only the adjuvant. Each animal was given 12 µg of each antigen together with 36 µg of EtxB at each occasion. On day 28, the mice were anaesthetized and infected with *Streptococcus equi* subsp. *equi* by intranasal administration of 10 µl of bacteria containing $1x10^6$ CFU (colony forming units).

Weight loss versus time is shown in Figure 10; on days 2, 3, 4, 5, and 8, the p-values were 0.04, 0.017, 0.005, 0.003, and 0.002, respectively. Nasal growth versus time is shown in Figure 11; on days 4 to 8, the p-value was <0.001. IgA antibody titers in BAL/NW and sera were determined by ELISA. Briefly, microtiter plates (Costar) were coated with 10 µg/ml of FNZN, SFSC1, and EAG4B. Serial dilutions of BAL/NWs were added to the wells. After washing, goat anti mouse IgA conjugated with horse radish peroxidase (Dako) was added. To develop the color reaction, OPD tablets (Dako) were added. From Figure 12 it is evident that BAL/NW samples from the animals contained IgA. Moreover, IgA against SFSC1 and EAG4B was also found in serum samples from some of the animals (data not shown).

Another group of mice (n=15) was immunized intranasally with SEC2.16 using Matrix (Ref. 20) as adjuvant and a control group was given the Matrix adjuvant alone (n=15). The animals were subjected to challenge infection with subsp. *equi*. The number of animals that died or lost more than 15% weight were different in the two groups; five vaccinated mice and 8 non-vaccinated died (Figure 18). This is not a statistically significant difference but is a tendency implying an effect of SEC2.16. It is likely that it would give a synergistic effect together with any or all of the other antigens mentioned above. Growth of subsp. *equi* in the noses was also studied. More bacteria were isolated from non-vaccinated mice than from vaccinated mice. On day 10, the score was 2.1 and 1.8 (on a scale from 0-3) in these groups, again implying an effect by vaccination with SEC2.16.

Example 12. Immunization of horses

Horses with no previous history of strangles and with low antibody titers against FNZ, SFS and EAG were selected. Four groups (A-D) of horses with three horses in each group were immunized as follows: A) EAG4B, FNZN and SFSC1 with EtxB as adjuvant, and given both intranasally and subcutaneously; B) As in group A but given only intranasally; C) as in A but without EtxB as adjuvant; and D) Only EtxB, and no antigens, given both

intranasally and subcutaneously. Each dose consisted of 35 μg of each antigen and 100 μg of EtxB in a volume of 2 ml for intranasal and/or subcutaneous immunization. Immunizations were given on days 0, 14, 28 and 42.

Serum samples were taken and analysed as follows: Microtiter wells (Costar) were coated overnight with 100 μl FNZN, SFSC1, or EAG4B, at 10 μg per ml overnight in phosphate buffered saline (PBS). The plates were then blocked with 2% (w/v) bovine serum albumin (BSA) for 1 hour at 37°C. After washing, horse sera were added to wells at a 20-fold dilution, followed by 2-fold serial dilutions. The plates were washed after 1 hour of incubation at 37°C. After washing, detection of antibody binding was performed with antibodies, diluted 1000x, against horse IgG, raised in rabbit and conjugated with HRP (Sigma Chemical Co). Development of a colorimetric reaction was achieved with OPD tablets (Dako, Denmark). Absorbance was determined spectrophotometrically at 492 nm.

In this way, IgG antibodies in sera of the immunized horses were determined. The results are shown in Figure 14. The log dilution of sera required to give an absorbance at a cut off value of 1.0 was calculated for each individual serum sample. Mean values (n=3) with standard errors are shown. Samples taken before (day 0) and after (day 56) immunizations are shown. The horses were divided into four groups A) (white bars): antigens + EtxB given both s.c. and i.n.; B) (striped bars): as group A but immunization only i.n.; C) (gray bars): As in group A but omitting EtxB; D) (black bars): control group given only EtxB both i.n. and s.c.

Example 13. Determination in ELISA of antibody titers from horses with or without a history of previous strangles

Serum samples were taken from horses (n=16) without any previous or present signs of strangles, as well as from horses (n=10) with clinical signs of strangles and positive results from cultivation of *S. equi*. Microtiter wells (Costar) were coated overnight with 100μl FNZN, SFSC1, EAG4B, SEC2.16 or SCLC1 at 10 μg per ml overnight in phosphate buffered saline (PBS). The plates were then blocked with 2% (w/v) BSA for 1 hour at 37°C. After washing, horse sera were added to wells at a 20-fold dilution, followed by 2-fold serial dilutions. The plates were washed after 1 hour incubation at 37°C. Detection of antibody binding was performed after washing, with antibodies, diluted 1000x, against horse IgG, raised in rabbits and conjugated with HRP (Sigma Chemical Co). Development of a colorimetric reaction was achieved with OPD tablets (Dako, Denmark). Absorbance was determined spectrophotometrically at 492 nm. The log10 dilution of sera, which was required to give an absorbance at a cut-off value of 1.0, was calculated for each individual serum

sample. Mean values of the log10 dilutions, with standard errors, are shown. For comparing normal vs. strangles sera against FNZN, SFSC1, EAG4B, SEC2.16 and SCLC1, the p-values are <0.001, 0.02, <0.0001, 0.02, and <0.0001, respectively.

In Figure 15, the results from a comparison of serum antibody titers from horses with or without strangles are shown. The designation n denotes normal horses without and the designatin s denotes horses with strangles. The log dilution of sera required to give an absorbance at a cut off value of 1.0 was calculated for each individual serum sample. Mean values with standard errors are shown. For comparing normal vs. strangles sera against FNZN, SFSC1, EAG4B, SEC2.16, and SCLC1, the p-values are <0.001, 0.02, <0.0001, 0.02 and < 0.0001, respectively.

Example 14. Determination of IgA antibody titers from, immunized horses

Horses were divided into four groups (A-D) and were immunized as described in Example 12. Antigen-specific IgA in nasal wash samples from immunized horses were determined by an indirect ELISA. Monoclonal antibody K129-3E7 (2 μg/ml) against equine IgA was used to detect bound IgA in the samples followed by use of a goat anti-mouse immunoglobulin HRP conjugate (2 μg/ml). Coating concentrations of antigens were 4 μg/ml. Background values (from no-antigen plates) were subtracted. All samples were analyzed in triplicate. Antigen-specific IgA ELISA data is expressed relative to the total IgA ELISA data in order to correct for nasal secretion dilution during the nasal wash procedure. Total IgA in nasal wash samples was determined by a capture ELISA using two different monoclonal antibodies specific to equine IgA. One was used to coat wells and the other was biotinylated and was detected with the use of a streptavidin-HRP conjugate. The concentrations of monoclonal antibody and streptavidin-HRP were optimized so that none of these was limiting in the assay. Samples were analysed in triplicate. In Figure 16, the Absorbance values obtained for IgA against FNZN and EAG4B are shown.

In Figure 16, IgA antibodies in nasal washings of immunized horses are shown. Mean absorbance values (n=3) in ELISA from groups A-D are shown. The horses were divided into four groups; A) antigens + EtxB given both s.c. and i.n.; B) as group A but immunization only i.n.; C) as in group A but omitting EtxB; D) control group given only EtxB both i.n. and s.c. Light gray bars: samples from day 0 and dark gray bars from day 56.

Example 15. Determination of kinetics for development of IgG in immunized horses.

Horses were immunized as described in Example 12. On days 0, 14, 28 and 42, samples were taken on these days and on day 56 as well and analyzed as described elsewhere. The horses were immunized with EAG4B, FNZN and SFSC1 with EtxB, by both intranasal and subcutaneous route.

The antibody titers against EAG4B found in these (three) horses are shown in Figure 17 as a function of time.

Example 16. Immunogenicity of protein SEC

Mice were immunized intranasally with SEC2.16, with (n=5) or without Matrix (n=5) using 20 µg per mouse of both SEC2.16 and Matrix. Serum samples were taken before and after 4 immunizations, with two-week intervals between. Pre-immune sera did not contain any detectable antibodies against SEC2.16. SEC2.16 without any adjuvant gave significant antibody responses in absence of Matrix. In presence of Matrix even better antibody titers were obtained as shown in Figure 19.

Example 17. Comparison of immunization with different antigens followed by challenge infection

Mice were immunized with proteins FNZN+SFSC1+EAG4B with EtxB (n=8) or with EAG4B with EtxB (n=10) as described in Example 11. Another group was given EtxB alone as control (n=9). The mice were subjected to challenge with subsp. *equi* and their weight change was monitored. As expected, the control group lost more weight than the groups that were given antigen, which confirms the protective effect of vaccination. The infected mice were left for 14 days. Animals in the group, which was given FNZN+SFSC1+EAG4B with EtxB as adjuvant regained weight from day 10, when average weight loss was approx. 10%, whereas on day 14, average loss was 7%. On the other hand, mice vaccinated with EAG4B with EtxB as adjuvant, kept loosing weight and on day 14 weight loss in this group was 12.5%. The difference in weight loss on day 14 implies that supplying EAG4B together with other antigens, such as SFSC1 and/or FNZN improves protection. However, the difference is not significant due to small sample size. (No data shown)

REFERENCES

1. **Courtney, H. S., Y. Li, J. B. Dale, and D. L. Hasty.** 1994. Cloning, sequencing and expression of a fibronectin/fibrinogen-binding protein from group A streptococci. Infect. Immun. **62**:3937-3946.

2. **Cue, D., P. E. Dombek, H. Lam, and P.P. Cleary.** 1998. *Streptococcus pyogenes serotype* M1 encodes multiple pathways for entry into human epithelial cells. Infect. Immun. **66**:4593-4601.

3. **Barnham, M., A. Ljunggren, and M. McIntyre.** 1987. Human infection with *Streptococcus zooepidemicus* (Lancefield group C): three case reports. Epidem. Inf. **98**;183.-190

4. **Galán, J.E., and J. F. Timoney.** 1988. Immunologic and genetic comparison of *Streptococcus equi* isolates from the United States and Europe. J. Clin. Microbiol. **26**:1142-1146.

5. **Hynes, T. O.** 1990. Fibronectins. Springer Verlag, New York.

6. **Engvall, E., E. Ruoslahti, and J. M. Miller.** 1978. Affinity of fibronectin to collagen of different genetic types and to fibronogen. J. Exp. Med. **147**:1584-1595.

7. **Patti, J. M., Jonsson, H., Guss, B., Switalski, L.M., Wiberg, K., Lindberg, M., and Höök, M.** (1992) Molecular characterization and expression of a gene encoding a *Staphylococcus aureus* collagen adhesin. *J. Biol. Chem.* **267**:4766-4772.

8. **Jonsson, H., Lindmark, H., and Guss. B.** (1995) A protein G related cell surface protein in *Streptococcus zooepidemicus. Infect Immun* **63**:2968-2975.

9. **Lindmark, H., Jacobsson, K., Frykberg, L., and Guss, B.** (1996) Fibronectin-binding protein of *Streptococcus equi* subspecies *zooepidemicus. Infect Immun* **64**:3993-3999.

10. **Jacobsson, K., Jonsson, H., Lindmark, H., Guss, B., Lindberg, M., and Frykberg. L.** (1997) Shot-gun phage display mapping of two streptococcal cell-surface proteins. *Microbiol Res.* **152**:1-8.

11. **Lindmark, H., Jonsson, P., Olsson-Engvall, E., and Guss, B.** (1999) Pulsed-field gel electrophoresis and distribution of the genes *zag* and *fnz* in isolates of *Streptococcus equi. Res Vet Sci.* 66:93-99.

12. **Lindmark, H., and Guss, B.** (1999) SFS, a novel fibronectin-binding protein from *Streptococcus equi,* inhibits the binding between fibronectin and collagen. *Infect. Immun.* 67: 2383-2388.

13. **Lindmark, H.** (1999) Characterization of adhesive extracellular proteins from *Streptococcus equi.* (Doctoral thesis) Acta Universitatis Agriculturae Sueciae, Agraria 139. ISBN 91-576-5488-3

14. **Lindmark, H., Nilsson, M., and Guss, B.** (2001) Comparison of the fibronectin-binding protein FNE from *Streptococcus equi* subspecies *equi* with FNZ from *S. equi* subspecies *zooepidemicus* reveals a major and conserved difference. 69: 3159-3163.

15. **Schneewind, O., Fowler, A. and Faull, K.F.** (1995) Structure of the cell wall anchor of surface proteins in *Staphylococcus aureus*. Science 268:103-106.

16. **Anton Mayr et al.** Handbuch der Shutzimpfungen in der Tiermedizin.3.3.4. p. 196-200. Verlag Paul Parey. Berlin and Hamburg. 11984

17. **Elson, C.O., and M.T. Dertzbaugh**. 1999. p. 817-838, Nucosal immunology, 2nd ed. Of Academic Press, New York, N.Y.

18. **Winner et al.** 1991. Infect. Immun. 59:997-982

19. **Ogra et al.** Clinical Microbiology Reviews, Apr. 2002, p. 430-445.

20. **B. Morein and Karin Lövgren Bengtsson** 1998, 76:295-299. Immunology and Cellbiology

21. Hanski, E., and M.G. Caparon. 1992. Protein F, a fibonectin-binding protein, is an adhesin of the group A streptococcus *Streptococcus pyogenes*. Proc. Natl. Acad. Sci. USA **89**:6172-6176.

22. Hanski, E., P.A. Horwitz, and M.G. Caparon. 1992. Expression of protein F, the Fibronectin-binding protein of *Streptococcus pyogenes* JRS4, in heterologous streptococcal and enterococcal strains promotes their adherence to respiratory epithelial cells. Infect. Immun. **60**:5119-5125.

23. Jadoun, J., V. Ozeri, E. Burstein, E. Skutelsky, E. Hanski, and S. Sela.1998. Protein F1 required for efficient entry of Streptococcus pyogenes into epithelial cells. J. Infect. Dis. **178**:147-158.

24. Molinri, G., S. R. Talay, P. Valentin-Weigand, M. Rohde, and G.S. Chhatwal. 1997. The fibronectin-binding protein of *Streptococcus pyogenes* SfbI, is involved in the internalization of group A streptococci by epithelial cells. Infect. Immun. **65**:1357-1363.

25. Lannergård, J., Frykberg, L. and Guss B. (2003) CNE, a collagen-binding protein of Streptrococcus equi. FEMS Microbiol. Lett. 222:69-74

26. WO 92/07002

27. WO 00/37496